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Citation for published version:
Fei, Y, Pyott, D & Molnar, A 2021, 'Temperature modulates virus-induced transcriptional gene silencing via secondary small RNAs', *New Phytologist*. https://doi.org/10.1111/nph.17586

Digital Object Identifier (DOI):
10.1111/nph.17586

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
New Phytologist

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Research

Temperature modulates virus-induced transcriptional gene silencing via secondary small RNAs

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Received: *25 May 2021*
Accepted: *17 June 2021*

New Phytologist (2021)
doi: 10.1111/nph.17586

Key words: DNA methylation, epigenetic modification, post-transcriptional gene silencing, secondary siRNAs, temperature, transcriptional gene silencing, transgenerational effect, virus-induced gene silencing.

Summary

- Virus-induced gene silencing (VIGS) can be harnessed to sequence-specifically degrade host transcripts and induce heritable epigenetic modifications referred to as virus-induced post-transcriptional gene silencing (ViPTGS) and virus-induced transcriptional gene silencing (ViTGS), respectively. Both ViPTGS and ViTGS enable manipulation of endogenous gene expression without the need for transgenesis.
- Although VIGS has been widely used in many plant species, it is not always uniform or highly efficient. The efficiency of VIGS is affected by developmental, physiological and environmental factors. Here, we use recombinant Tobacco rattle viruses (TRV) to study the effect of temperature on ViPTGS and ViTGS using *GFP* as a reporter gene of silencing in *N. benthamiana* 16c plants.
- We found that unlike ViPTGS, ViTGS was impaired at high temperature. Using a novel mismatch-small interfering RNA (siRNA) tool, which precisely distinguishes virus-derived (primary) from target-generated (secondary) siRNAs, we demonstrated that the lack of secondary siRNA production/amplification was responsible for inefficient ViTGS at 29°C. Moreover, inefficient ViTGS at 29°C inhibited the transmission of epigenetic gene silencing to the subsequent generations.
- Our finding contributes to understanding the impact of environmental conditions on primary and secondary siRNA production and may pave the way to design/optimize ViTGS for transgene-free crop improvement.

Introduction

RNA silencing is an evolutionarily conserved mechanism that regulates gene expression in eukaryotes and meditates defence against invasive nucleic acids such as transposons, transgenes and viruses (Baulcombe, 2004). RNA silencing can result from two genetically and mechanistically distinct pathways. Post-transcriptional gene silencing (PTGS) involves the degradation or translational repression of RNA targets, while transcriptional gene silencing (TGS) involves cytosine methylation of DNA at target loci (Baulcombe, 2004; Brodersen & Voinnet, 2006; Borges & Martienssen, 2015). In addition to regulating endogenous gene expression, RNA silencing plays a crucial role in plants as a potent antiviral defence mechanism (Ding, 2010; Pumplin & Voinnet, 2013; Guo *et al.*, 2019). The presence of virus genomes in plant cells triggers an antiviral RNA-silencing response whereby 21–24 nucleotide (nt) small interfering RNA (siRNA) molecules are produced to direct the silencing of the viral genome. For RNA viruses, double-stranded replication intermediates or secondary structures in the genome (Molnar *et al.*, 2005) trigger the production of siRNAs, while for DNA viruses double-stranded RNA arising from bidirectional transcription of the virus genome is likely to be the main trigger for siRNA production (Pooggin, 2013). Double-stranded RNA (dsRNA) is processed into siRNAs by the action of plant Dicer-like (DCL) proteins (Bernstein *et al.*, 2001) and these siRNA molecules are loaded into ARGONAUTE (AGO) proteins to form an RNA-induced silencing complex (RISC). In the case of RNA viruses, the RISC disables the virus by cleavage of the RNA genome in a manner analogous to PTGS, while DNA viruses are repressed by DNA methylation, analogous to TGS mechanisms (Ghoshal & Sanfacon, 2015; Guo *et al.*, 2019). This innate immune strategy has been applied to develop a method for silencing endogenous genes in plants, known as virus-induced gene silencing (VIGS) (Ruiz *et al.*, 1998). To silence a specific plant gene, a fragment of the target gene is inserted into a suitable virus vector. During viral replication, virus-derived siRNAs (vsiRNAs) that are homologous to the target locus are generated and can thus knock down expression of the target gene through RNA silencing mechanisms.

Virus-induced gene silencing is a powerful tool for studying plant functional genomics, especially for species for which stable genetic transformation is not possible (Burch-Smith *et al.*, 2004; Dommes *et al.*, 2019). Many VIGS vectors are available for gene silencing in various plant species (both dicots and monocots) (Senthil-Kumar & Mysore, 2011; Yuan *et al.*, 2011; N. Liu *et al.*,

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2016). To date, more than 30 plant viruses have been modified as VIGS vectors for inducing gene silencing in plants. Four of these have been shown to induce heritable TGS, including Potato virus X (PVX), Tobacco rattle virus (TRV), Cucumber mosaic virus (CMV) and Apple latent spherical virus (ALSV) (Jones *et al.*, 1999; Jones *et al.*, 2001; Kanazawa *et al.*, 2011; Kon & Yoshikawa, 2014; Bond & Baulcombe, 2015).

Most VIGS strategies rely on PTGS, which is associated with siRNA-directed mRNA cleavage, resulting in degradation of target mRNA in the host. In some instances, the targeting of an mRNA by primary siRNAs can further trigger the production of secondary siRNAs. In virus-induced post-transcriptional gene silencing (ViPTGS), virus-derived primary siRNAs that have sequence similarity to the host RNA can serve as primers to amplify dsRNA precursors from host RNA transcript using the host RNA-dependent RNA polymerases (RDRs) (Brodersen & Voinnet, 2006; Voinnet, 2008). This triggers the production of secondary siRNAs from the regions upstream and downstream of the initial target site, a phenomenon called transitivity (Vaistij *et al.*, 2002; Voinnet, 2008; Aregger *et al.*, 2012). Secondary siRNAs can move between cells and even long distances (from source to sink) through the vascular system to facilitate movement of the silencing signal (Schwach *et al.*, 2005; Brosnan & Voinnet, 2011). Potentially, the amplification of secondary siRNAs from transitive silencing would further enhance ViPTGS.

Some VIGS vectors can also cause TGS by inducing DNA methylation at the target gene promoter sequence to suppress gene expression (Jones *et al.*, 2001; Otagaki *et al.*, 2011; Bond & Baulcombe, 2015). This process is initiated by siRNAs that guide core proteins of RNA silencing to target homologous DNA sequences and direct cytosine methylation in all sequence contexts (i.e. CG, CHG and CHH, where H = A, C or T) in plants (Law & Jacobsen, 2010; Baulcombe & Dean, 2014; Gallego-Bartolome, 2020). Such siRNA-guided epigenetic modification of the genome is referred to as RNA-directed DNA methylation (RdDM) and is recognized as the initial step of *de novo* DNA methylation in plants (Matzke & Mosher, 2014). Unlike animals, DNA methylation in plants is not erased in every generation, but rather it can be trans-generationally maintained (Heard & Martienssen, 2014). Maintenance of DNA methylation in plants depends on the cytosine sequence context and is associated with different DNA methyltransferases (Zhang *et al.*, 2018). Symmetrical methylation at CG and CHG can be maintained independently of siRNAs during DNA replication by METHYLTRANSFERASE1 (MET1) and CHROMOMETHYLASE 3 (CMT3), respectively, both of which copy the methylation onto the opposite cytosine residue of the daughter stand (Law & Jacobsen, 2010). However, cytosine methylation in an asymmetric CHH context cannot be maintained during DNA replication and therefore requires persistent *de novo* methylation by DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) (Zhong *et al.*, 2014).

In previous studies, some VIGS vectors have been shown to induce heritable TGS of endogenous genes in *Arabidopsis*, *Petunia* and *Solanum* plants (Kanazawa *et al.*, 2011; Bond & Baulcombe, 2015). Thus, virus-induced transcriptional gene silencing (ViTGS) is one of the novel methods for induction of stable transgenerational epigenetic modifications in plants with the potential to modify the expression of agronomically important traits without transgenesis. Although VIGS protocols have been improved and widely used in many plant species, gene silencing is not always uniform or highly efficient. Virus-induced gene silencing depends on several factors including the developmental stage of plants, virus multiplication rate (virus replication, spreading and titre) and environmental conditions (Senthil-Kumar & Mysore, 2011a,b). Among these, environmental factors (particularly temperature) play a critical role in VIGS. Temperature can influence VIGS from two aspects: a plant aspect and a virus aspect. Temperature can dramatically affect the activities of antiviral defence response in plants (Szittya *et al.*, 2003; Chellappan *et al.*, 2005; Zhang *et al.*, 2012). It has been shown in previous studies that key factors of antiviral silencing pathways, such as AGOs, DCLs and RDRs, are temperature-dependent (Zhang *et al.*, 2012; Qu *et al.*, 2005; J. Liu *et al.*, 2016). Higher temperature promotes this defence response by enhancing the activities of the core proteins of RNA silencing to increase siRNA accumulation, and lower temperature inhibits RNA silencing-mediated defence by limiting the biogenesis of siRNA molecules (Velázquez *et al.*, 2010; J. Liu *et al.*, 2016; Ma *et al.*, 2016; Szittya *et al.*, 2003). From the virus aspect, viruses also require a specific temperature range to successfully infect plants. Most of plant viruses are able to infect plants at 20–25°C, but some viruses are low-temperature-dependent and only infect plants below 20°C (such as viruses infecting winter crops) (Andika *et al.*, 2013; Hull, 2013; Yang *et al.*, 2018). Virus-induced gene silencing is dependent on the plant–virus interaction. Therefore, it is important to understand the comprehensive effects of temperature on the efficacy of VIGS and to optimize the environmental conditions for VIGS vectors and plant species.

Materials and Methods

Plant material

Nicotiana benthamiana (line 16c) (Ruiz *et al.*, 1998), expressing the *Green Fluorescent Protein* (*GFP*) under the control of the *Cauliflower mosaic virus 35S* (*35S*) promoter, were used in this study. *Nicotiana benthamiana* 16c plants were grown in the growth chamber (Sanyo/Panasonic) at 22°C with a 16 h : 8 h, light : dark photoperiod.

Construction of TRV-based VIGS vectors

The recombinant TRV constructs were as described previously (Fei *et al.*, 2021). Briefly, the 120 nt fragments from the *35S* promoter (−208 to −89; Otagaki *et al.*, 2011), the *GFP* coding sequence (+364 to +483; Ruiz *et al.*, 1998) and their variant carrying single-nucleotide substitutions (SNSs) at every 10 nt within the fragment were inserted into the *Sma*I site of the TRV RNA2 vector (pTRV2) (Dinesh-Kumar *et al.*, 2003) to generate constructs TRV-35S, TRV-GFP, TRV-35S-2M and TRV-GFP-2M.

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Viral inoculations

The binary vectors containing TRV RNA1 (pTRV1), TRV RNA2 (pTRV2) and recombinant pTRV2s were transformed into *Agrobacterium tumefaciens* (strain GV3101:pMP90 + pSOUP). To generate infectious recombinant TRV sap, fully expanded leaves of 3- to 4-wk-old wild-type *N. benthamiana* plants were coinfiltrated with *Agrobacterium* carrying pTRV1 or any form of pTRV2 mixed at a 1 : 1 ratio (Ratcliff *et al.*, 2001). The systemic leaves of infiltrated plants were collected at 7 d post-inoculation (dpi) and ground in 1 mM sodium phosphate buffer (pH 7.0) to obtain viral sap. To infect *N. benthamiana* 16c plants, three fully expanded leaves of 3- to 4-wk-old *N. benthamiana* 16c plants were rub-inoculated with 10 µl of viral sap, using aluminium oxide as an abrasive.

After viral inoculations, the infected plants were transferred to growth chambers at constant temperature of 15, 22 and 29°C under 16 h : 8 h, light : dark cycles (Fig. 2; Supporting Information Fig. S1). In the repeated experiments, the infected plants were kept at 22°C for 2 d and then shifted to 22 and 29°C under 16 h : 8 h, light : dark cycles (Figs 5b, S7).

Imaging of GFP fluorescence

Green fluorescence was observed using a handheld mercury UV lamp (UVP Blak-Ray™ B-100AP High-Intensity UV Inspection Lamp, Upland, CA, USA). Photographs were taken using a Canon G16 camera. Camera exposure settings were f/3.2, ranging from 3 to 6 s, depending on the intensity of GFP fluorescence and distance from the plant.

Quantitative real-time PCR (qRT-PCR)

Total nucleic acid (TNA) was isolated from systemic leaves of the plants using phenol-chloroform extraction as previously described (White & Kaper, 1989). DNA was removed by treating 3 µg of TNA with Turbo DNase (Ambion) according to the manufacturer's instructions. A 1 µg amount of RNA was used to synthesise cDNA with random hexamers and Superscript II (Invitrogen). Quantitative RT-PCR analysis were performed using SYBR Green I Master Mix on a LightCycler® 480 instrument (Roche). *Nicotiana benthamiana EF1a* was used as the internal control. Gene-specific primers used for qRT-PCR are listed in Table S1. The following cycling conditions were used for all reactions: 95°C, 5 min > (95°C, 10 s > 60°C, 10 s > 72°C, 15 s) × 45.

DNA methylation analysis using bisulfite sequencing

DNA samples were extracted from *c.* 100 mg of leaf tissue using GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich). Approximately 400 ng of DNA was bisulfite-treated with the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instruction. The recovered DNA was amplified by PCR with One Taq Hot Start DNA polymerase (NEB) using gene-specific oligonucleotides (Table S1). The amplified products were cloned into the pGEM-T Easy vector (Promega) and were subsequently transformed to *Escherichia coli* (DH5alpha strain) cells. Eight to 16 clones from each sample were sequenced. Bisulfite-converted sequences were aligned to the corresponding sequences using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). DNA methylation patterns were analysed using the CyMate software (Hetzl *et al.*, 2007). The Wilson score was calculated as shown in (Henderson *et al.*, 2010; Jullien *et al.*, 2012).

Small RNA cloning and bioinformatics analysis

Small RNA libraries were prepared from 1 µg of TNA using the Illumina TruSeq Small RNA Library Prep Kit (Illumina, San Diego, CA, USA). Small RNA libraries were single-end sequenced on the Illumina HiSeq 2500 platform with read lengths of 50 bases at Edinburgh Genomics (Edinburgh, UK). Sequence analysis was performed using the Geneious software (v.11.1.4; http://www.geneious.com). Briefly, adaptor sequences were trimmed from fastq reads. The remaining sequences in the size range 21–24 nt were mapped either to recombinant TRV RNA2s or to the transgenic *N. benthamiana* 16c T-DNA+partialTn5393 locus (GenBank accession no. KY464890) and its derivatives, where target sequences were modified according to the SNSs that were introduced into the recombinant TRV trigger (TRV-35S-2M and TRV-GFP-2M). All alignments only allowed perfectly matching small RNAs. The small RNA sequencing data are available in the ArrayExpress database under accession number E-MTAB-9394.

Results

Temperature exhibits significant effects on ViTGS

To investigate how temperature affects VIGS, we chose TRV as an inducer of gene silencing and *GFP* driven constitutively by the *35S* promoter as a reporter gene in transgenic *N. benthamiana* 16c (Fig. 1a). Recombinant TRVs were generated by introducing 120 nt fragments from the *35S* promoter (TRV-35S) and from the *GFP* coding region (TRV-GFP) (Fig. 1b). By using equally sized inserted fragments of 120 nt, we ensured a comparable substrate for the production of virus-derived small RNAs. The TRV-35S recombinant virus was used to induce TGS, while a TRV-GFP was generated to trigger PTGS. Plants inoculated with a TRV empty vector (TRV-WT) were used as viral infection controls. *Nicotiana benthamiana* 16c plants were rub-inoculated with the viral sap of TRV-35S, TRV-GFP or TRV-WT. After rub inoculation, plants were kept at different temperatures (15, 22 and 29°C) and were monitored at regular intervals under UV light.

We observed that temperature dramatically affected the antiviral defence responses of the plants. Symptomatically, most severe TRV symptoms were presented at 15°C, whereas symptoms were milder at higher temperatures (Figs 2a, S1a). To assess viral RNA accumulation more accurately, qRT-PCR was performed using systemic leaves of infected plants kept under different growth temperatures. In agreement with the phenotypes observed, a much lower accumulation of viral RNA was detected at 29°C

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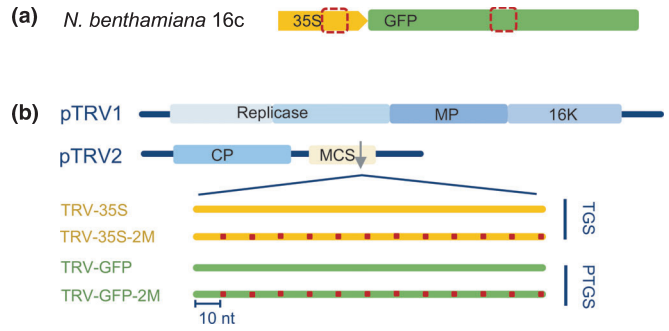

Fig. 1 Tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) vectors used for silencing of the *GFP* transgene. (a) Schematic diagram of the *CaMV 35S* promoter-driven *GFP* reporter gene in *Nicotiana benthamiana* 16c plant. The virus-targeted regions are indicated by red rectangles in the diagram. (b) TRV was used as the VIGS vector. A 120 nt fragment of the *35S* promoter (−208 to −89) and the *GFP* coding sequence (+364 to +483) was cloned into TRV-RNA2 to induce transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS), respectively. Single-nucleotide substitutions (SNSs) were introduced to the 120 nt *35S* sequence and the 120 nt *GFP* sequence at 10 nucleotide intervals to generate TRV-35S-2M and TRV-GFP-2M, respectively. The positions of SNSs are indicated by red boxes. TRV-RNA1 was used along with recombinant TRV-RNA2 to generate functional TRV particles. MCS, multiple cloning sites; CP, coat protein; MP, movement protein.

compared with either of the lower temperatures for samples collected at 7, 14 and 21 dpi (Figs 2c, S1b). This is consistent with the previous findings that antiviral silencing is temperature-dependent, such that higher temperatures are associated with an increased production of viral small RNAs and a concomitant reduction in genomic viral RNA (Szittya *et al.*, 2003; Chellappan *et al.*, 2005; Havelda *et al.*, 2005; Qu *et al.*, 2005; Velázquez *et al.*, 2010; Ma *et al.*, 2016).

We then assessed the VIGS efficacy under these three growth temperatures. At 22°C, strong *GFP* silencing was detected in both TRV-35S- and TRV-GFP-infected *N. benthamiana* 16c plants at 7 dpi, using qRT-PCR to measure the abundance of *GFP* mRNA (Fig. S1b, green bars). At 15°C, both TRV-35S- and TRV-GFP-infected plants showed delayed *GFP* silencing (Fig. S1a,b). Complete *GFP* silencing (where *GFP* expression was near the threshold of detection by qRT-PCR) was detected at 14 dpi for plants maintained at 15°C (Fig. S1b), 1 wk later than the appearance of complete silencing for plants maintained at 22°C. This suggests that the low temperature slowed down the progression of antiviral RNA silencing. Although *GFP* silencing was delayed at 15°C, it was as persistent as that at 22°C. Strong *GFP* silencing was maintained at 21 dpi for TRV-35S- and TRV-GFP-infected plants kept at 15 and 22°C (Fig. 2a,b). Intriguingly, at the higher temperature of 29°C, antiviral RNA silencing exhibited a more dynamic response. At early stages of infection (7 dpi), *GFP* silencing rapidly developed on upper systemic leaves in both TRV-35S- and TRV-GFP-infected plants (Fig. S1a). However, TRV-35S-induced *GFP* silencing failed to spread further throughout the plants or to the newly emerging leaves. GFP fluorescence was completely restored in TRV-35S-infected plants at later times after inoculation (Fig. 2a). By contrast, TRV-GFP-infected plants exhibited stronger and persistent *GFP* silencing phenotypes (Fig. 2a). Quantification of the *GFP* expression by qRT-PCR confirmed the phenotypic observations. A full reduction of *GFP* expression could be detected in TRV-GFP-infected plants kept at 29°C at 21 dpi, whereas TRV-35S-infected plants showed similar *GFP* expression level to TRV-WT-infected plants (Fig. 2b). These results reveal that ViTGS and ViPTGS act differently at high temperatures.

Virus-induced transcriptional gene silencing is associated with siRNA-mediated DNA methylation in the target promoter sequence and results in transcriptional gene silencing (Jones *et al.*, 2001). To gain insight into the DNA methylation status of the targeted *35S* promoter sequence, bisulfite sequencing was used to analyse the degree of cytosine methylation in the systemic leaves of TRV-35S-infected plants. As shown in Figs 2(d), S2 and Table S2, a high degree of DNA methylation was detected in the *35S* promoter (−208 to −89) region of TRV-35S-infected plants kept at 15 and 22°C, but only a few cytosine residues of the promoter were methylated in infected plants kept at 29°C. The bisulfite sequencing results were correlated with the silencing phenotype of the plants and confirmed that TRV-35S could direct stable and constant TGS at 15 and 22°C, but not at the higher temperature of 29°C.

Inefficient ViTGS cannot transmit to the subsequent generations

It has been shown in previous studies that ViTGS triggers sequence-specific DNA methylation and heritable transcriptional gene silencing (Jones *et al.*, 1999, 2001; Otagaki *et al.*, 2011). To test the effect of temperature on transgenerational epigenetic gene silencing, we analysed the virus-free progeny of TRV-35S-infected plants that had been maintained at different temperatures. We refer to the plants that were challenged by TRV-35S to initiate ViTGS as the S_0 generation and their immediate progeny as the S_1 generation. S_1 seedlings were germinated at 22°C and were screened under UV light for *GFP* silencing phenotypes. Silencing phenotypes were observed in the progeny of the S_0 plants that had been maintained at either 15 or 22°C, indicating that ViTGS established in the parental lines kept at these temperatures was heritable (Fig. 3a). By contrast, no silencing phenotype was observed in the progeny of the S_0 plants that were maintained at 29°C, suggesting that inefficient establishment of ViTGS in the parental plants kept at this temperature prevented the inheritance of the silencing phenotype. Detailed analysis of *GFP* expression (Fig. 3b) and promoter DNA methylation (Figs 3c, S2; Table S2) exhibited a strong inverse correlation in the progeny and reduced *GFP* expression was always accompanied by high degrees of symmetric cytosine methylation (CG and CHG).

The maintenance of virus-induced TGS is not impaired at high temperature

Virus-induced transcriptional gene silencing-mediated DNA methylation can be heritable across mitotic and meiotic cell divisions by virtue of RdDM maintenance mechanisms (Law & Jacobsen, 2010; Heard & Martienssen, 2014). We were therefore

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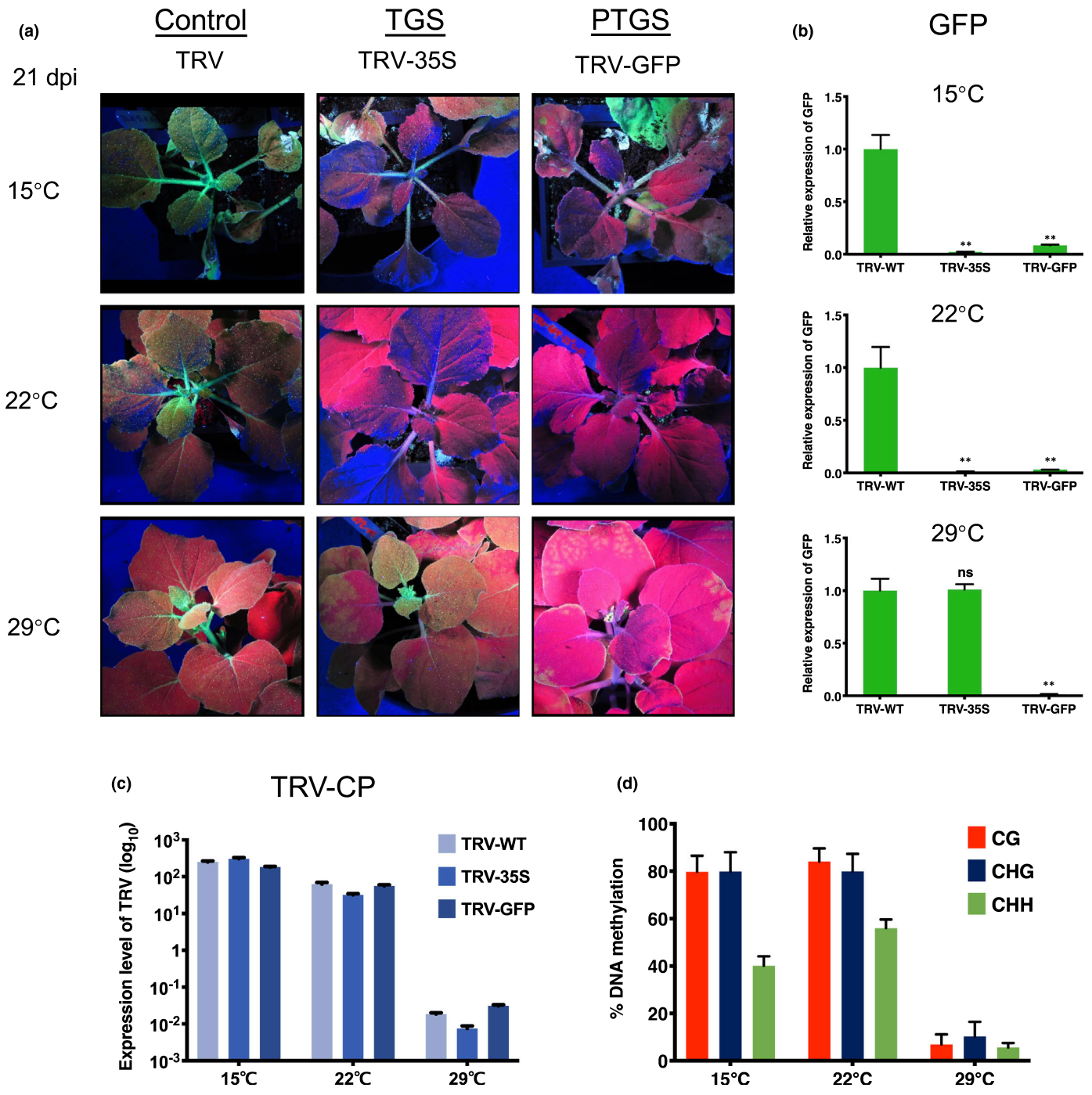

Fig. 2 Effect of temperature on Tobacco rattle virus (TRV)-induced *GFP* silencing in *Nicotiana benthamiana* 16c plants. (a) Silencing phenotype of *N. benthamiana* 16c plants infected with transcriptional gene silencing (TGS)-inducing TRV-35S and post-transcriptional gene silencing (PTGS)-inducing TRV-GFP kept at 15/22/29°C. Wild-type TRV was used as a viral infection control. The plants were photographed at 21 d post-inoculation (dpi) under UV light. *GFP* silencing results in red fluorescence owing to the autofluorescence of Chl. (b, c) Relative expression of *GFP* and TRV coat protein (TRV-CP) in the systemic leaves of the recombinant TRV-infected plants at 21 dpi. Samples were taken from second and third systemic leaves of infected plants at 21 dpi. Expression levels were determined by quantitative reverse transcription polymerase chain reaction. Error bars show the SEM of three biological replicates. Each replicate was composed of samples from three plants pooled together (two leaves per plant). **, $P < 0.01$; ns, nonsignificant (Student's *t*-test). (d) DNA methylation status of the target *35S* promoter (from −208 to −89) in the systemic leaves of TRV-35S-infected *N. benthamiana* 16c plants at 21 dpi. Samples were taken from second and third systemic leaves of infected plants at 21 dpi. The DNA methylation was assessed by bisulfite sequencing. The red, blue and green bars show the percentage of methylated cytosine at the CG, CHG and CHH sites, repetitively. Results were obtained from three biological replicates. Each replicate was composed of samples from three plants pooled together (two leaves per plant). Raw data are available in Supporting Information Fig. S2 and Table S2. Error bars represent a confidence interval with 95% confidence limits (Wilson score interval; see details in Table S2).

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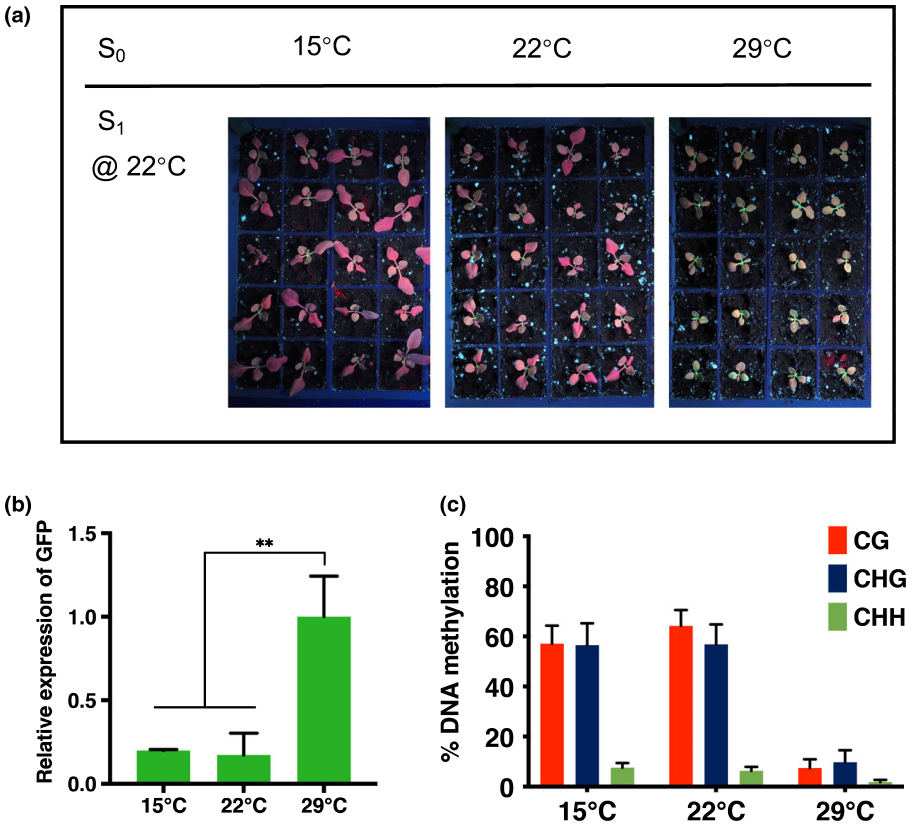

Fig. 3 Inheritance of virus-induced transcriptional gene silencing (ViTGS) (a) Progeny (S_1) of Tobacco rattle virus (TRV)-35S-infected *Nicotiana benthamiana* 16c plants (S_0) that had been maintained at 15/22/29°C. S_1 seedlings were germinated and maintained at 22°C. Plants were photographed under UV light at 21 d after germination. (b) Relative expression of *GFP* in the 7-wk-old S_1 plants. Samples were taken from second and third leaves of the 7-wk-old S_1 plants. Expression levels were determined by quantitative reverse transcription polymerase chain reaction. Error bars show the SEM of three biological replicates. Each replicate was composed of samples from three plants pooled together (two leaves per plant). **, $P < 0.01$ (Student's *t*-test). (c) DNA methylation status of the target *35S* promoter (from −208 to −89) in the 7-wk-old S_1 plants. Samples were taken from second and third leaves of the 7-wk-old S_1 plants. The DNA methylation was assessed by bisulfite sequencing. The red, blue and green bars show the percentage of methylated cytosine at the CG, CHG and CHH sites, repetitively. Results were obtained from three biological replicates. Each replicate was composed of samples from three plants pooled together (two leaves per plant). Raw data are available in Supporting Information Fig. S2 and Table S2. Error bars represent a 95% interval (Wilson score interval; see details in Table S2).

interested to test whether the temperature-dependent effects on ViTGS seen in our silencing system could involve disruptions to the maintenance and/or epigenetic inheritance of ViTGS. To this end, we interrogated the *GFP* silencing phenotypes in the progeny of TRV-35S-infected plants. Seeds were collected from TRV-35S-infected plants kept at 22°C. After germination, plants were shifted to 29°C and assessed for the maintenance of the silencing phenotype. The S_1 progeny of the TGS plants usually exhibit a variable degree of *GFP* silencing. To avoid this side-effect of assessing the maintenance of DNA methylation in TGS, all seedlings were maintained at 22°C for 4 wk, and were then sorted into groups based on the degree of inherited *GFP* silencing (Fei *et al.*, 2021). The progeny within the strongest *GFP* silencing group were selected and shifted to 29 or 22°C (Fig. 4). Interestingly, shifting the S_1 plants to 29°C did not appear to affect the *GFP* silencing phenotype as it was comparable to that of the S_1 plants kept at 22°C. This indicates that exposing S_1 plants to the higher temperature of 29°C did not disrupt the maintenance of ViTGS in this generation. To stringently test for any temperature-dependent effects on the maintenance of ViTGS we analysed the *GFP* silencing phenotypes of the subsequent (S_2) generation. All S_2 plants were grown at the same temperature of 22°C to ensure that any differences in *GFP* silencing phenotypes would result from heritable effects of ViTGS caused by the differential temperature treatments of S_1 plants. Progeny of TRV-WT-infected plants were kept under the same conditions and used as controls (Fig. 4). The degree of *GFP* silencing was identical for all S_2 plants, regardless of whether they were derived from S_1 plants kept at 22 or 29°C, suggesting that the heritable maintenance of ViTGS was not affected by the temperature treatment in the S_1 generation (Fig. 4). Interestingly, in agreement with these observations, we found that elevated growth temperature did not impair the maintenance of transgene-induced TGS (Figs S3–S5; Table S3; Methods S1–S2; Notes S1).

Distinguishing primary and secondary small RNA by using specifically designed inducer sequences

As we did not discern any effects of temperature on the maintenance of ViTGS or transgene-induced TGS, we reasoned that the

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differences in ViTGS phenotypes observed at different temperatures must arise from processes upstream of the initiation of RdDM. Moreover, the dramatic difference in the long-term effects on *GFP* silencing after incubation at 29°C between the TRV-35S- and TRV-GFP-challenged plants led us to speculate that there could be differences in the extent of siRNA amplification between the PTGS and TGS systems (Fig. 2a). In other words, we reasoned that infection with TRV-GFP could stimulate the production of secondary siRNAs from the plant-derived *GFP* transcript, resulting in the persistence of a siRNA pool even after recovery from the viral infection as a result of the raised antiviral response at 29°C. By contrast, in the case of TRV-35S-challenged plants, the production of secondary siRNAs from the *35S* promoter sequence may not be as efficient. Therefore, the pool of siRNAs directing TGS in our ViTGS system may result mainly from primary (virus-derived) siRNAs and, to a much lesser extent, from secondary (derived from the *35S* transgene) siRNAs. Thus, the TGS silencing response would not persist after recovery from viral infection at 29°C. To test this hypothesis, we employed a mismatch-siRNA system that can precisely

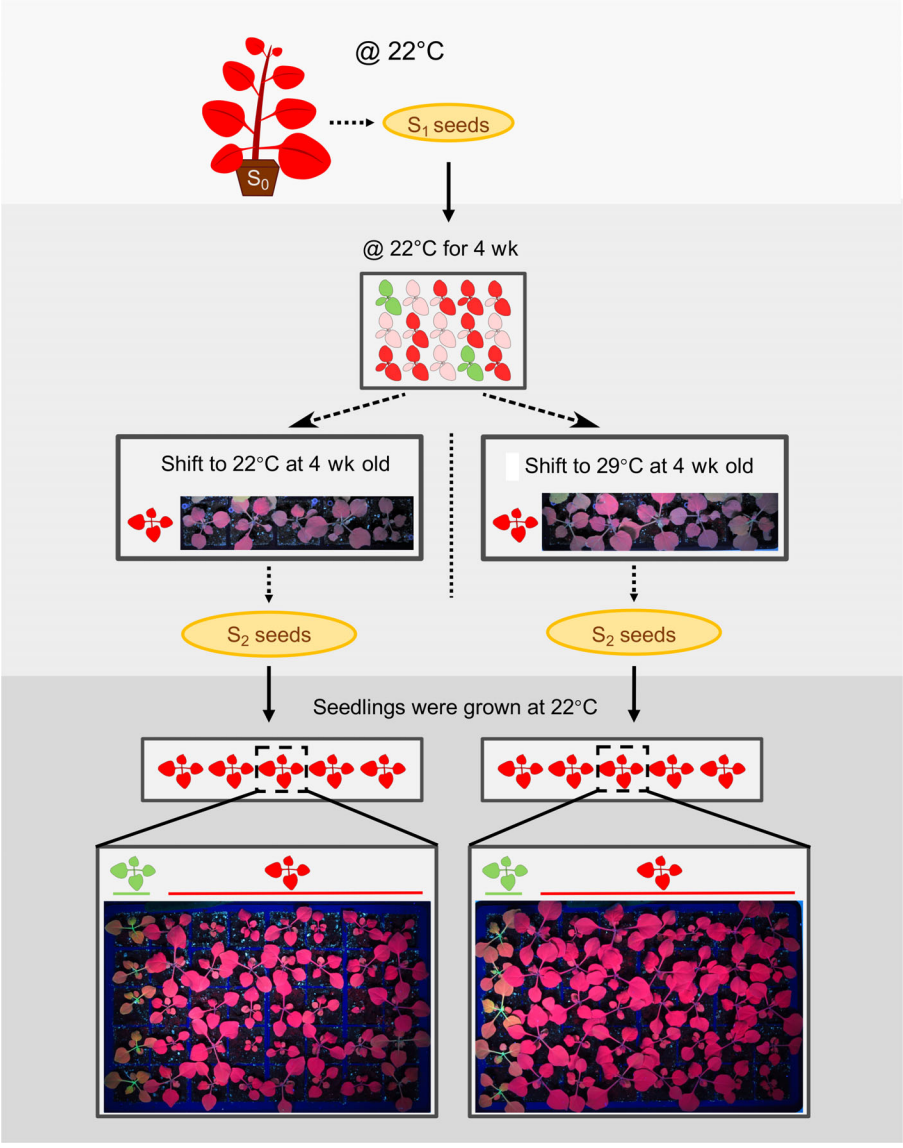

Fig. 4 Inheritance of virus-induced transcriptional gene silencing (ViTGS) is not affected by high temperature. Schematic diagram of experimental design to assess the maintenance of transgenerational epigenetic gene silencing in the progeny of Tobacco rattle virus (TRV)-35S-infected plants at the different temperatures. The S_1 seeds were collected from the TRV-35S-infected plants (S_0) exhibiting *GFP* silencing that had been maintained at 22°C. The S_1 seedlings were germinated and maintained at 22°C for 4 wk. According to the degree of inherited *GFP* silencing, the 4-wk-old S_1 plants with strongest *GFP* silencing were selected and shifted to 22 and 29°C. The S_2 seeds were collected from five independent S_1 plants (lines) from each temperature group. All S_2 seedlings (35 seedlings from each line) were germinated and maintained at 22°C for 3 wk, and were then assessed for the maintenance of the *GFP* silencing phenotype under UV light. Progeny of wild-type TRV-infected plants kept under the same conditions were used as controls and indicated under the green lines.

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differentiate primary and secondary siRNAs via high-throughput sequencing (Fei *et al.*, 2021). Briefly, we generated a variant of TRV-35S, named TRV-35S-2M, carrying SNSs at every 10 nucleotides within the 120 nt *35S* promoter sequence (Fig. 1b). Hence, virus-derived primary siRNAs processed from TRV-35S-2M contain at least two distinct SNSs to the *35S* target sequence, whereas secondary siRNA amplified from the endogenous target sequence in plants will contain no SNSs (Fig. 5a). This two-SNS design strategy allows virus-derived primary siRNAs to be distinguished with more certainty from secondary siRNAs. TRV-GFP-2M was designed with a similar approach targeting a 120 nt segment from the *GFP* coding region to distinguish siRNAs in ViPTGS (Figs 1b, 5a).

Virus-induced transcriptional gene silencing and ViPTGS were distinct in their phenotype under high temperature whereas no phenotypic variation was seen at 22°C. Therefore, we focused on

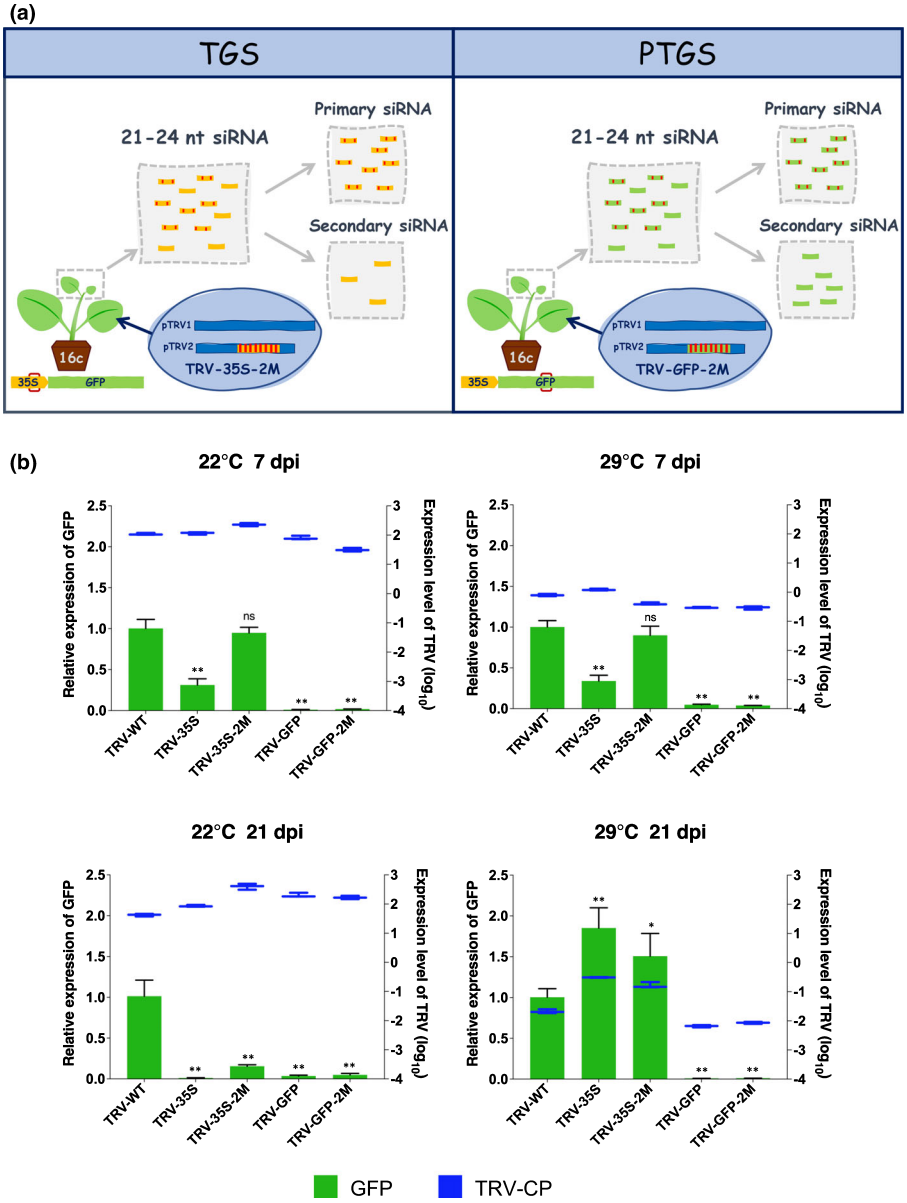

Fig. 5 Identifying primary and secondary small RNAs by employing specifically designed RNA silencing inducer sequences. (a) Schematic diagram of experimental design to distinguish primary and secondary small RNAs. Small RNAs containing single-nucleotide substitutions (SNSs) (red) are primary small interfering RNAs (siRNAs) generated from the virus, while siRNAs without SNSs are secondary siRNAs amplified from the *GFP* transgene. TGS, transcriptional gene silencing; PTGS, post-transcriptional gene silencing. (b) Time-course of *GFP* and Tobacco rattle virus coat protein (TRV-CP) expression in the recombinant TRV-infected *Nicotiana benthamiana* 16c plants at 22 and 29°C. The green bars and blue lines represent the relative expression of *GFP* and TRV-CP in the recombinant TRV-infected plants, respectively. Samples were collected from the systemically infected leaves (second and third leaves) at 7 and 21 d post-inoculation (dpi). Expression levels were determined by quantitative reverse transcription polymerase chain reaction. Error bars show the SEM of three biological replicates. Each replicate was composed of samples from three plants pooled together (two leaves per plant). *, $P < 0.05$; **, $P < 0.01$; ns, nonsignificant (Student's *t*-test).

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the 22 and 29°C growth conditions in the following experiment. We noted that directly shifting infected plants to 29°C also promotes antiviral RNA silencing in the local inoculated leaves. This limits the virus replication in the inoculated tissues, and consequently affects virus uploaded into the phloem for systemic viral infection. Thus, we assessed whether increasing the viral replication in the local inoculated tissue could allow more virus to be uploaded into the systemic leaves and further extend the systemic silencing at high temperature. After inoculation, the TRV-WT-infected plants were held at 22°C for 2 d before shifting to 29°C to allow the virus to replicate in the local tissues. Quantitative RT-PCR results showed that a 2 d incubation at 22°C significantly increased the viral accumulation in both inoculated and systemic leaves in the early stages of infection compared with those immediately shifted to 29°C (Fig. S6). Hence, we applied the 2 d incubation at 22°C before shifting to 22 or 29°C in the following experiment. Although viral RNA for the plants kept at 29°C was increased after 2 d incubation at 22°C, the amount of virus in the systemic leaves of plants kept at 29°C was still *c.* 100-fold less than for plants kept at 22°C by 7 dpi (Fig. 5b, right vs left panels, blue lines). This is reminiscent of previous studies reporting enhanced RISC-based resistance at high growth temperatures (Zhang *et al.*, 2012; J. Liu *et al.*, 2016) and indicates that antiviral RNA silencing is a response that acts both locally and systemically. Importantly, there was no significant difference in the abundance of viral RNA between recombinant TRVs (TRV-35S and TRV-GFP) and their variants (TRV-35S-2M and TRV-GFP-2M).

Samples were collected at an early (7 dpi) and a late time point (21 dpi) to measure the *GFP* expression using qRT-PCR (Fig. 5b, green bars). At 22°C, *GFP* expression in TRV-35S-infected plants showed an approximately three-fold decrease compared with TRV-WT-infected plants at 7 dpi, and was below the threshold of detection at 21 dpi (Fig. 5b). TRV-35S-2M, carrying SNSs to its target sequence, could also trigger *GFP* silencing, but the progression was much slower than TRV-35S infection. Strong *GFP* silencing could only be observed in systemic leaves of TRV-35S-2M-infected plants at 21 dpi (Figs 5b, S7). At 29°C, TRV-35S-infected plants showed more expanded TGS with 2 d incubation at 22°C compared with the TRV-35S-infected plants shifted to 29°C immediately after infection. We observed clear *GFP* silencing in the systemic leaves at 7 dpi and detected a similar reduction of *GFP* mRNA as that of 22°C (Figs 5b, S7). However, *GFP* expression was fully restored in the newly emerging leaves of TRV-35S-infected plants at 21 dpi (Figs 5b, S7). Unlike ViTGS, ViPTGS did not show much difference between 22 and 29°C. At both temperatures, TRV-GFP-infected plants revealed a full reduction of *GFP* expression at 7 dpi, and the *GFP* silencing phenotype was persistent over the course of infection (Fig. 5b). Interestingly, the SNSs in TRV-GFP-2M did not impair its silencing efficiency. TRV-GFP-2M-infected plants had similar *GFP* suppression level as TRV-GFP. From these experiments, we conclude that the SNSs presented in siRNA can reduce its targeting efficiency, especially in ViTGS. The different rates of ViTGS and ViPTGS progression might indicate their differences in silencing signal amplification/secondary siRNA production.

The secondary siRNA production affects the maintenance of ViTGS at high temperature

To investigate whether the different efficiencies of ViTGS and ViPTGS were a result of difference in secondary siRNA production, we sequenced siRNAs from virus-infected plants kept at 29°C at 7 and 21 dpi. We first assessed the quality of our siRNA libraries by aligning siRNA sequences to the TRV RNA2 genome. As expected, siRNAs were unevenly distributed along the viral genome, but their distribution patterns were very similar across all samples at 7 dpi (Fig. 6). Moreover, we found that the abundance of virus-specific siRNAs was associated with TRV infection. There were more virus-derived siRNAs accumulated at 7 dpi, whereas only a few siRNAs were detected at 21 dpi, which was in accordance with our qRT-PCR results (Figs 5b, 6). Strikingly, we observed a cluster of *GFP*-specific siRNAs aligned to the TRV-RNA2-GFP genome at 21 dpi (Fig. 6; Table S4). We suspect that these *GFP*-specific siRNA accumulations could be secondary siRNAs amplified from the *N. benthamiana* 16c plants' *GFP* sequence.

As a control, we also sequenced siRNAs from wild-type TRV-infected *N. benthamiana* 16c plants and aligned them both to the *35S* promoter and the *GFP* coding region (Fig. S8). We detected a single siRNA that matched the *35S* promoter and very low abundance of siRNAs that mapped evenly along the *GFP* coding region, probably as a result of transgene transcript degradation. These data indicate that siRNAs triggered by 120 nt segments in the recombinant TRV genomes are the predominant source of siRNA, resulting in the silencing of *GFP* in our experimental system.

To separate the virus-derived primary siRNAs from the target-generated secondary siRNAs, we separated the reads according to SNS content by aligning them to the target *GFP* locus (Fig. 7a). In the control TRV-GFP-infected 16c plants, where primary and secondary siRNAs could not be distinguished, we detected high amounts of *GFP*-specific siRNAs of all sizes (21–24 nt), indicating that multiple Dicers could act on the siRNA precursor (Fig. 7a, left panel). These siRNAs were not restricted to the 120 nt segment of the *GFP* target region, but extended to the rest of the *GFP* sequence (Fig. 7a, left panel). This indicated that ViPTGS was associated with transitivity and the production of secondary siRNAs. The same siRNA distributions were observed for siRNAs from TRV-GFP-2M-infected plants, indicating that SNSs did not affect the production of secondary siRNAs (Fig. 7a, middle and right panels). Interestingly, we observed a gap in TRV-GFP-2M primary siRNA alignment at 21 dpi, which was a result of virus-derived primary siRNA (GFP-2M-specific siRNAs) elimination. These results indicate that ViPTGS is associated with large amount of secondary siRNA production, and these secondary siRNAs are sufficient to maintain the ViPTGS in the absence of primary siRNAs.

In contrast to ViPTGS, siRNAs were aligned exclusively to the targeted 120 nt *35S* region and did not extend into the rest of the *35S* sequence in TRV-35S-infected plants (Fig. 7b). The same siRNA distribution was observed for primary siRNAs from TRV-35S-2M-infected plants at 7 dpi. In addition, only a few

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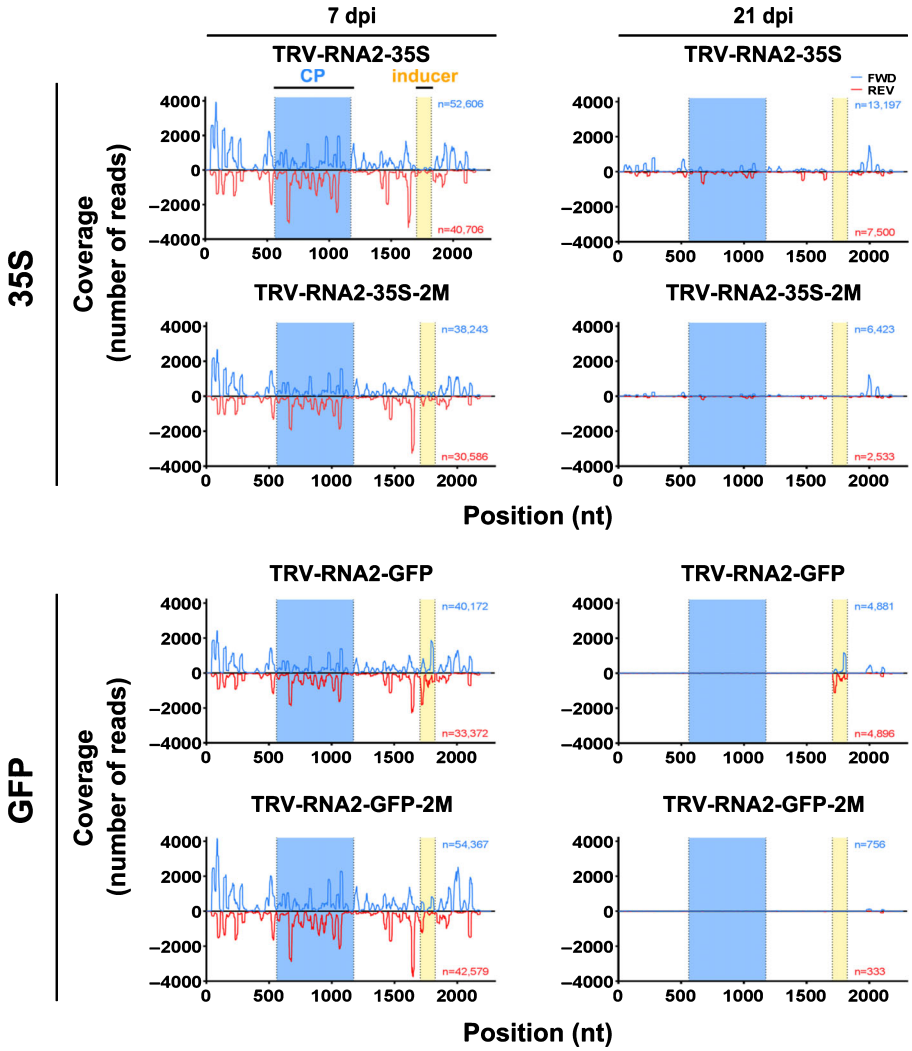

Fig. 6 Analysis of virus-derived small RNAs in recombinant-Tobacco rattle virus (TRV)-infected plants at 29°C. The 21–24 nt small RNAs were aligned to the corresponding recombinant TRV RNA2. The small RNA reads mapped to the plus and minus strand are shown in blue and red lines, respectively. The coat protein (CP) and the silencing-inducer sequences are highlighted as blue and yellow boxes, respectively. The total number of small RNAs mapped to each recombinant TRV RNA2 are also indicated in the graph. dpi, d post-inoculation.

secondary siRNAs were identified in TRV-35S-2M-infected plants, and these were in very low abundance. These data suggest that there was no or very limited transitivity (spreading of siRNAs beyond the target site) at the *35S* locus as a result of the lack of secondary siRNA production. To exclude the possibility that the limited secondary siRNA amplification in ViTGS was affected by temperature, we examined the *35S*-specific siRNA alignment in the TRV-35S- and TRV-35S-2M-infected 16c plants kept at 22°C (Fei *et al.*, 2021). In line with the data at 29°C, promoter-associated siRNAs (inducing TGS) appear to be

Fig. 7 The secondary small interfering RNA (siRNA) production affects the maintenance of recombinant-Tobacco rattle virus (TRV)-induced *GFP* silencing at high temperature. (a) Small RNA analysis of *Nicotiana benthamiana* 16c plants infected with post-transcriptional gene silencing (PTGS)-inducing viruses TRV-GFP-2M and TRV-GFP maintained at 29°C. Small RNAs from TRV-GFP-2M-infected plants were sorted according to single-nucleotide substitution (SNS) content to yield primary (containing SNSs) and secondary siRNAs (lacking SNSs). Primary siRNAs from TRV-GFP-2M-infected plants mapped to the 120 nt *GFP*-2M target site were indicated by the black arrow (middle panel). Secondary siRNAs from TRV-GFP-2M-infected plants were aligned to the target site and adjacent regions (right panel). Small interfering RNAs from TRV-GFP-infected plants aligned to the *GFP* coding sequence were used as controls (left panel). The target sites are highlighted by dotted lines. Small interfering RNA reads mapped to the plus strand are presented as positive values, and those mapped to the minus strand are presented as negative values. The 21–24 nt siRNAs are indicated with different colours. (b) Small RNA analysis of *N. benthamiana* 16c plants infected with transcriptional gene silencing (TGS)-inducing viruses TRV-35S-2M and TRV-35S maintained at 29°C. Small RNAs from TRV-35S-2M-infected plants were separated into primary and secondary siRNAs according to SNS content, and were aligned to the 120 nt 35S-2M sequence and to the *35S* promoter sequence, respectively. Small RNAs from TRV-35S-infected plants aligned to the *35S* promoter sequence were used as controls (left panel). Labelling as in (a). dpi, d post-inoculation.

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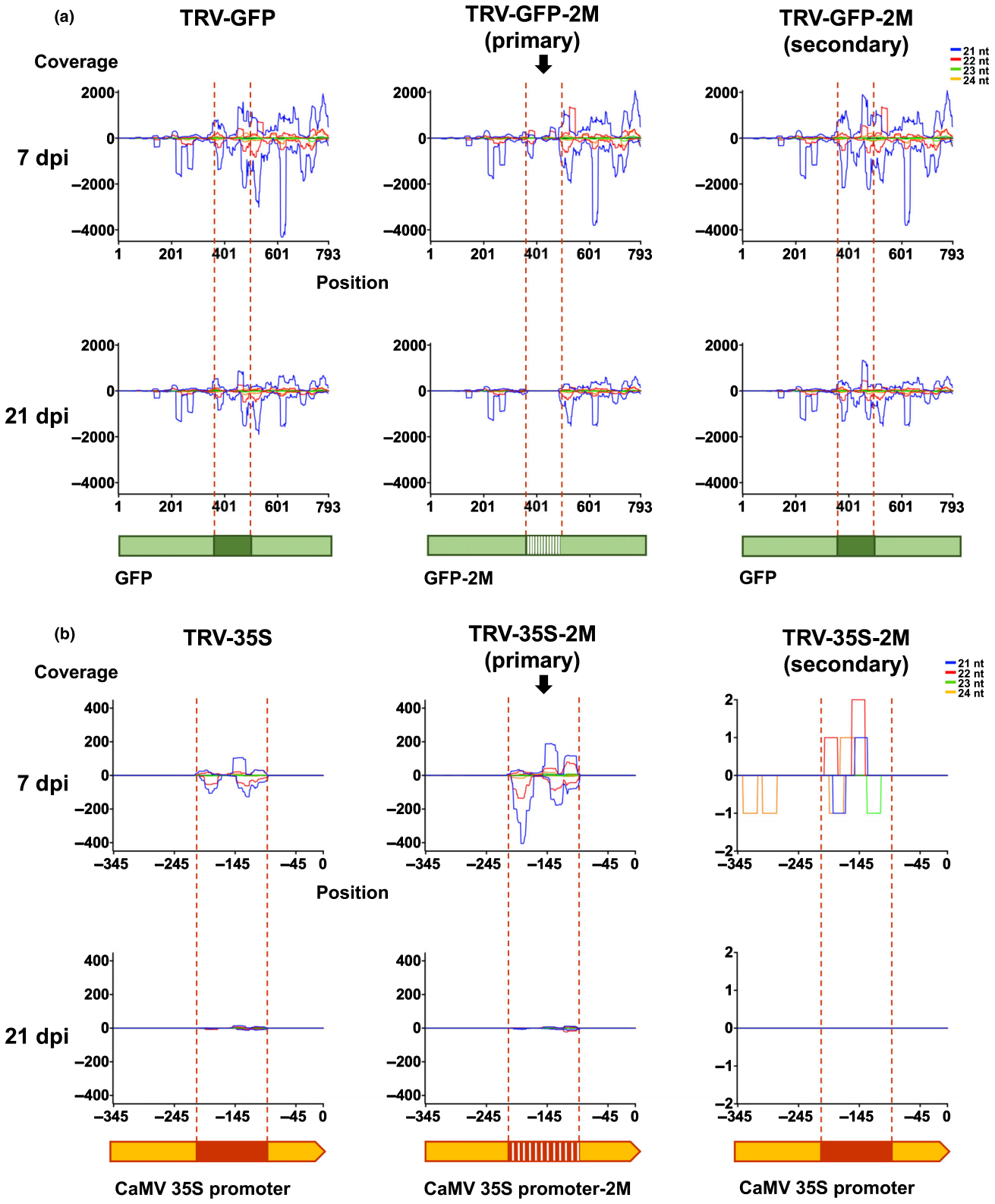

exempt from secondary siRNA amplification at 22°C (Fei *et al.*, 2021). In the absence of abundant secondary siRNAs, primary siRNAs took the essential role of supporting ViTGS. Hence, when most of virus-derived primary siRNAs were eliminated as a result of the virus clearance, ViTGS could not be further maintained (Figs 5b, 7b).

As DCL2-dependent 22 nt siRNAs have been implicated in the systemic spread of PTGS (Taochy *et al.*, 2017; Chen *et al.*, 2018),

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we analysed the accumulation and relative abundance of *GFP*-specific siRNAs in the primary and secondary siRNA pool by mapping siRNAs to the 120 nt *GFP*-2M and the 120 nt *GFP* sequence, respectively, in TRV-GFP-2M-infected 16c plants at 7 dpi (Fig. S9a,b). Interestingly, we observed a temperature-dependent shift from primary to secondary siRNA production (Fig. S9b) and an increase in 21 nt siRNA level among secondary siRNAs, probably as a result of DCL4 activity. However, the 21/22 nt siRNA ratio was similar between 22 and 29°C (Fig. S9a,b), suggesting that the rapid onset of PTGS at ambient and high temperatures is rather attributed to an increase in the number of mixed-sized siRNAs than to the accumulation of a specific siRNA class.

From these experiments, we conclude that when viral RNAs are cleared from the infected plants by the effective antiviral RNA silencing at high temperature, it also removes the source of virus-derived primary siRNAs. Unlike ViPTGS, ViTGS is not able to generate a large number of secondary siRNAs and depends solely on its primary siRNAs, resulting in the loss of silencing phenotype at later times of the infection process.

Discussion

In this study, we compared the efficiency of inducing gene silencing between ViTGS and ViPTGS, which has not been directly compared in previous studies. Using recombinant TRV viruses to trigger TGS or PTGS of a transgenic *35S-GFP* reporter gene, we found that ViTGS can achieve similar effectiveness as ViPTGS at 22°C. However, ViTGS is more sensitive to the temperature changes. Unlike ViPTGS, ViTGS does not persist at later times after inoculation at high temperatures. This is a result of the differences in the molecular mechanisms underlying the ViTGS and ViPTGS phenomena (Fig. 8). Virus-induced post-transcriptional gene silencing can generate a large number of secondary siRNAs to reinforce silencing, whereas ViTGS does not produce any secondary siRNAs and depends solely on primary siRNAs (Figs 7, 8). High temperatures trigger the hyperactive antiviral RNA silencing which can effectively clear viral RNAs. This limits the production of primary siRNAs and hence reduces the silencing phenotype of ViTGS.

Secondary siRNAs have been implicated in playing an important role in plant-virus interactions. They contribute to defence against viruses and can activate antiviral mechanisms ahead of virus invasion front to prime the uninfected cells (Schwach *et al.*, 2005; Melnyk *et al.*, 2011; Korner *et al.*, 2018). In our study, secondary siRNAs appear to enhance gene silencing triggered by primary siRNAs. Our data show that secondary siRNAs amplified from transgenic *GFP* mRNA persistently maintained the silencing phenotype of ViPTGS, even in the absence of primary

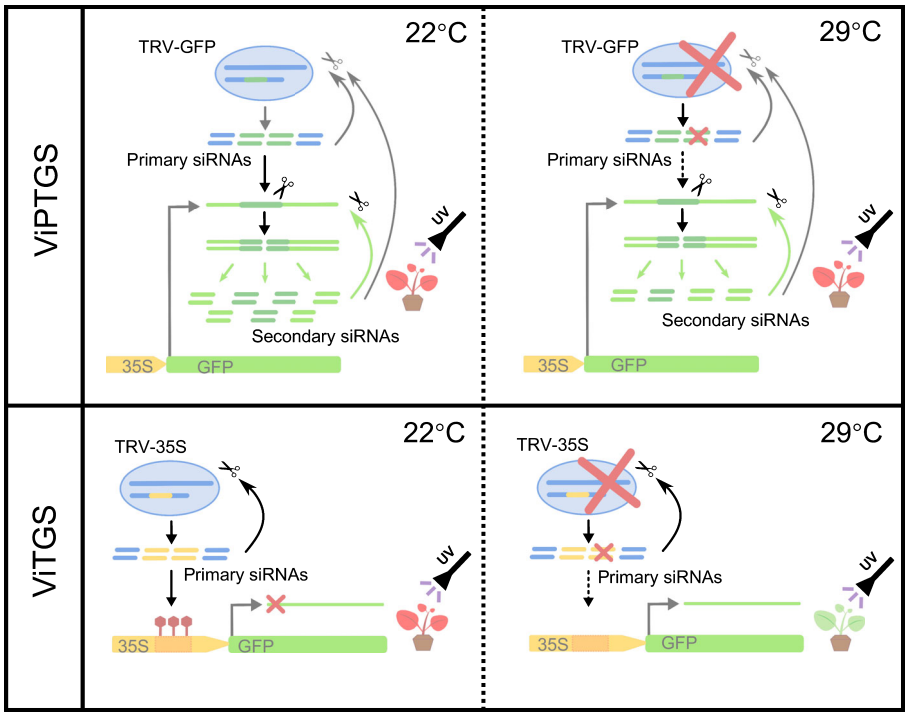

Fig. 8 Different molecular mechanisms of virus-induced post-transcriptional gene silencing (ViPTGS) and virus-induced transcriptional gene silencing (ViTGS). At 22°C, in ViPTGS, primary small interfering RNAs (siRNAs) derived from Tobacco rattle virus (TRV)-green fluorescent protein (GFP) mediate *GFP* mRNA cleavage, resulting in post-transcriptional gene silencing (PTGS) of *GFP*. The cleavage of *GFP* mRNA can further trigger the amplification of secondary siRNAs from the plant-derived *GFP* transcript. The production of secondary siRNAs enhances PTGS of *GFP* in *Nicotiana benthamiana* 16c plants. In ViTGS, primary siRNAs derived from TRV-35S direct DNA methylation at the target *35S* promoter sequence, resulting in transcriptional gene silencing (TGS) of *GFP*. Virus-induced transcriptional gene silencing (ViTGS) does not trigger secondary siRNA amplification, but mainly depends on virus-derived primary siRNAs. At the higher temperature of 29°C, plants exhibit effective antiviral RNA silencing responses to clear viral RNAs. This limits the production of virus-derived primary siRNAs. Unlike ViPTGS, ViTGS is not able to produce a large number of secondary siRNAs and only depends on its primary siRNAs, resulting in the loss of silencing phenotype at later times of the infection process. Solid and dotted arrows indicate full and reduced fluxes, respectively.

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siRNAs (Figs 5b, 7a). Massive amounts of secondary siRNAs were generated from the targeted site and adjacent regions (Fig. 7a). The amplification property of secondary siRNAs makes them more abundant than primary siRNAs. Thus, ViPTGS exhibits a more effective silencing ability compared with ViTGS. However, transitivity of secondary siRNAs depends on the target loci. A transgenic locus is prone to generate a large number of secondary siRNAs, whereas endogenous loci tend to have fewer secondary siRNAs (Vaistij *et al.*, 2002; Aregger *et al.*, 2012; Hartl *et al.*, 2017). This is probably because transgene transcripts might have some aberrant features (such as sequences or structures) that may be conducive to heightened recruitment of RDR proteins. This may explain why RNA silencing tends to be more effective in silencing transgenes than endogenous loci. In addition, we cannot rule out the possibility that if ViPTGS is used to target a gene that does not support secondary siRNA production, then the silencing phenotype would also be lost at high temperatures. This is more likely to happen in endogenous loci. The extent of secondary siRNA production at endogenous loci is not yet known, as tools to investigate this phenomenon by distinguishing primary and secondary siRNAs have not yet been developed. Now, with our mismatched-siRNA system, it is possible to assess the production of secondary siRNAs from targeted endogenous loci, and further reveal the efficiency of ViPTGS at endogenous loci.

Interestingly, we found that vsiRNAs targeting the *35S* promoter region caused transcriptional *GFP* silencing without secondary siRNAs production. Likewise, no secondary siRNAs were found when the nontranscribed *35S* enhancer region was targeted by vsiRNAs in geminivirus-induced gene silencing (Aregger *et al.*, 2012). These results indicate that the production of primary vsiRNAs is likely to be a major determinant for the efficacy of ViTGS. However, the production of secondary siRNAs has been reported for a transgene TGS system (Kanno *et al.*, 2008), indicating that the role of secondary siRNAs in ViTGS may be more relevant in certain cases. These differences may result from the silencing system, the target locus-specific factors or the origin of primary siRNAs. It is still not clear what the general mechanism of secondary siRNA production is at the nontranscribed promoter regions, especially at promoters of endogenous genes. Future studies using our mismatched-siRNA system to analyse the efficacy of ViTGS for different gene promoters (both endogenous and transgene) will probably identify additional information on secondary siRNA biogenesis from the nontranscribed promoter regions.

Temperature exhibits a great impact on RNA silencing. It has been well established that antiviral RNA silencing is more active with increased temperature (Szittya *et al.*, 2003; Chellappan *et al.*, 2005; Havelda *et al.*, 2005; Qu *et al.*, 2005; Velázquez *et al.*, 2010; Ma *et al.*, 2016). However, the effect of temperature on RNA silencing varies across different systems and organisms. For example, high temperature can inhibit sense transgene-mediated PTGS (S-PTGS) by degradation of SUPPRESSOR OF GENE SILENCING 3 (SGS3) protein and suppression of trans-acting siRNA biogenesis, and the heat-induced release of PTGS exhibits transgenerational epigenetic inheritance (Zhong *et al.*, 2013; J. Liu. *et al.*, 2019; N. Liu. *et al.*, 2019). In *Schizosaccharomyces pombe* and *Drosophila melanogaster*, silencing is most effective at lower temperatures (Allshire *et al.*, 1994; Lloyd *et al.*, 2003; Kloc *et al.*, 2008). These differences might be a result of divergent species evolving to have different optimal temperatures for the silencing response and small RNA biogenesis (Kloc & Martienssen, 2008). It is known that abiotic environmental stimuli, including heat, cold, drought, high salinity and UV radiation stress, can alter plant DNA methylation at individual loci or across the entire genome (Zhang *et al.*, 2018). Thus, we assessed whether the high temperature could disrupt the epigenetic inheritance of ViTGS by shifting the S_1 progeny of the TGS plants to 29°C. Interestingly, the high temperature does not alter the *GFP* silencing phenotype in those shifted plants or their subsequent (S_2) generation. This indicates that in the *35S-GFP* system, VIGS-induced DNA methylation as an epigenetic marker can be stably maintained at high temperature. Using an *Agrobacterium*-mediated transient assay, we also investigated the effect of temperature on transgene-induced TGS in the above reporter system. We detected strong *GFP* silencing and a high degree of promoter DNA methylation in TGS-inducer infiltrated leaves kept at 29°C (Figs S4, S5; Table S3), indicating that elevated temperature does not generally suppress TGS. This experiment also confirms that high temperature does not interfere with the maintenance of DNA methylation. Intriguingly, we found a high degree of corresponding DNA methylation in TRV-GFP-infected plants that were immediately transferred to 29°C after virus inoculation (Fig. S10; Notes S2), suggesting that the initiation of virus-induced RdDM is not impaired at high temperature when plenty of secondary siRNAs are around (Fig. 7a). However, further work is required to test the impact of a wider range of environmental stresses on the initiation and maintenance of DNA methylation at endogenous loci.

Epigenetic modification is known as a novel strategy to manipulate gene expression in plants and to potentially improve plant traits. It is different from conventional genetic modification techniques as it does not change the coding sequence of the plant genome. DNA methylation is an important and conserved epigenetic modification in eukaryotes. DNA methylation in plants can be artificially induced in several ways: siRNA-mediated DNA methylation (VIGS (Bond & Baulcombe, 2015), inverted repeat transgenes (Wakasa *et al.*, 2018; Zicola *et al.*, 2019)), using a programmable DNA-binding proteins to directly target methylation (zinc finger proteins (Johnson *et al.*, 2014; Gallego-Bartolome *et al.*, 2019) and CRISPR-dCas9 (Papikian *et al.*, 2019)). Our study uses VIGS technology to induce DNA methylation and TGS. We showed that once the ViTGS-mediated DNA methylation is well established in the parental lines, it will be inherited to subsequent generations. Such epigenetic gene silencing is persistent for several generations and appears to be unaffected by changes in the growth temperature. The establishment of the heritable silencing in the progeny correlated positively with the amount of virus and siRNAs in the parental lines (Bond & Baulcombe, 2015). Previous studies have suggested maintaining the silencing in the parental lines until seed maturity to promote the inheritance of the silencing (Senthil-Kumar & Mysore,

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2014). Owing to the particular feature of ViTGS (the limited transitivity of secondary siRNAs), it requires a sustained supply of virus-derived primary siRNAs to establish a complete TGS in the parental lines. Thus, an optimal temperature that allows continuous virus multiplication while at the same time ensuring active RNA silencing is critical to support the persistent establishment of TGS to enable heritable silencing.

Acknowledgements

YF was funded by the China Scholarship Council. DEP was supported by the BBSRC EastBio DTP award BB/J01446X/1 and the James Hutton Institute studentship. We would like to thank Prof. David Baulcombe for providing 16c *N. benthamiana* seeds, Prof. James Carrington for providing the pCB-p19, and Prof. George P. Lomonossoff for providing the pEAQ-GFP-*HT*.

Author contributions

AM designed the research; YF and AM performed the experiments. YF, DEP and AM analysed the data. YF, DEP and AM wrote the manuscript.

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Data availability

The data supporting the findings of this study are available within the article and its supporting information. The small RNA sequencing data have been deposited in the Array-Express database and can be accessed with the accession number E-MTAB-9394.

References

Allshire RC, Javerzat JP, Redhead NJ, Cranston G. 1994. Position effect variegation at fission yeast centromeres. *Cell* **76**: 157–169.

Andika IB, Sun L, Xiang R, Li J, Chen J. 2013. Root-specific role for *Nicotiana benthamiana* RDR6 in the inhibition of *Chinese wheat mosaic virus* accumulation at higher temperatures. *Molecular Plant-Microbe Interactions* **26**: 1165–1175.

Aregger M, Borah BK, Seguin J, Rajeswaran R, Gubaeva EG, Zvereva AS, Windels D, Vazquez F, Blevins T, Farinelli L *et al.* 2012. Primary and secondary siRNAs in geminivirus-induced gene silencing. *PLoS Pathogens* **8**: e1002941.

Baulcombe D. 2004. RNA silencing in plants. *Nature* **431**: 356–363.

Baulcombe DC, Dean C. 2014. Epigenetic regulation in plant responses to the environment. *Cold Spring Harbor Perspectives in Biology* **6**: a019471.

Bernstein E, Caudy AA, Hammond SM, Hannon GJ. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**: 363–366.

Bond DM, Baulcombe DC. 2015. Epigenetic transitions leading to heritable, RNA-mediated de novo silencing in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences, USA* **112**: 917–922.

Borges F, Martienssen RA. 2015. The expanding world of small RNAs in plants. *Nature Reviews Molecular Cell Biology* **16**: 727–741.

Brodersen P, Voinnet O. 2006. The diversity of RNA silencing pathways in plants. *Trends in Genetics* **22**: 268–280.

Brosnan CA, Voinnet O. 2011. Cell-to-cell and long-distance siRNA movement in plants: mechanisms and biological implications. *Current Opinion in Plant Biology* **14**: 580–587.

Burch-Smith TM, Anderson JC, Martin GB, Dinesh-Kumar SP. 2004. Applications and advantages of virus-induced gene silencing for gene function studies in plants. *The Plant Journal* **39**: 734–746.

Chellappan P, Vanitharani R, Ogbe F, Fauquet CM. 2005. Effect of temperature on geminivirus-induced RNA silencing in plants. *Plant Physiology* **138**: 1828–1841.

Chen W, Zhang X, Fan Y, Li B, Ryabov E, Shi N, Zhao M, Yu Z, Qin C, Zheng Q *et al.* 2018. A genetic network for systemic RNA silencing in plants. *Plant Physiology* **176**: 2700–2719.

Dinesh-Kumar SP, Anandalakshmi R, Marathe R, Schiff M, Liu Y. 2003. Virus-induced gene silencing. *Methods in Molecular Biology* **236**: 287–294.

Ding SW. 2010. RNA-based antiviral immunity. *Nature Reviews Immunology* **10**: 632–644.

Dommes AB, Gross T, Herbert DB, Kivivirta KI, Becker A. 2019. Virus-induced gene silencing: empowering genetics in non-model organisms. *Journal of Experimental Botany* **70**: 757–770.

Fei Y, Nyiko T, Molnar A. 2021. Non-perfectly matching small RNAs can induce stable and heritable epigenetic modifications and can be used as molecular markers to trace the origin and fate of silencing RNAs. *Nucleic Acids Research* **49**: 1900–1913.

Gallego-Bartolome J. 2020. DNA methylation in plants: mechanisms and tools for targeted manipulation. *New Phytologist* **227**: 38–44.

Gallego-Bartolome J, Liu W, Kuo PH, Feng S, Ghoshal B, Gardiner J, Zhao JM, Park SY, Chory J, Jacobsen SE. 2019. Co-targeting RNA polymerases IV and V promotes efficient *De Novo* DNA methylation in *Arabidopsis*. *Cell* **176**: 1068–1082.

Ghoshal B, Sanfacon H. 2015. Symptom recovery in virus-infected plants: Revisiting the role of RNA silencing mechanisms. *Virology* **479–480**: 167–179.

Guo Z, Li Y, Ding SW. 2019. Small RNA-based antimicrobial immunity. *Nature Reviews Immunology* **19**: 31–44.

Hartl K, Kalinowski G, Hoffmann T, Preuss A, Schwab W. 2017. RNAi-mediated endogene silencing in strawberry fruit: detection of primary and secondary siRNAs by deep sequencing. *Plant Biotechnology Journal* **15**: 658–668.

Havelda Z, Hornyik C, Válóczi A, Burgyán J. 2005. Defective interfering RNA hinders the activity of a tombusvirus-encoded posttranscriptional gene silencing suppressor. *Journal of Virology* **79**: 450–457.

Heard E, Martienssen RA. 2014. Transgenerational epigenetic inheritance: myths and mechanisms. *Cell* **157**: 95–109.

Henderson IR, Deleris A, Wong W, Zhong X, Chin HG, Horwitz GA, Kelly KA, Pradhan S, Jacobsen SE. 2010. The *de novo* cytosine methyltransferase DRM2 requires intact UBA domains and a catalytically mutated paralog DRM3 during RNA-directed DNA methylation in *Arabidopsis thaliana*. *PLoS Genetics* **6**: e1001182.

Hetzl J, Foerster AM, Raidl G, Mittelsten SO. 2007. CyMATE: a new tool for methylation analysis of plant genomic DNA after bisulphite sequencing. *The Plant Journal* **51**: 526–536.

Hull R. 2013. *Plant virology*. Cambridge, MA, USA: Academic press.

Johnson LM, Du J, Hale CJ, Bischof S, Feng S, Chodavarapu RK, Zhong X, Marson G, Pellegrini M, Segal DJ *et al.* 2014. SRA- and SET-domain-containing proteins link RNA polymerase V occupancy to DNA methylation. *Nature* **507**: 124–128.

Jones L, Hamilton AJ, Voinnet O, Thomas CL, Maule AJ, Baulcombe DC. 1999. RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. *The Plant Cell* **11**: 2291–2301.

Jones L, Ratcliff F, Baulcombe DC. 2001. RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. *Current Biology* **11**: 747–757.

Jullien PE, Susaki D, Yelagandula R, Higashiyama T, Berger F. 2012. DNA methylation dynamics during sexual reproduction in *Arabidopsis thaliana*. *Current Biology* **22**: 1825–1830.

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Kanazawa A, Inaba JI, Shimura H, Otagaki S, Tsukahara S, Matsuzawa A, Kim BM, Goto K, Masuta C. 2011. Virus-mediated efficient induction of epigenetic modifications of endogenous genes with phenotypic changes in plants. *The Plant Journal* **65**: 156–168.

Kanno T, Bucher E, Daxinger L, Huettel B, Bohmdorfer G, Gregor W, Kreil DP, Matzke M, Matzke AJ. 2008. A structural-maintenance-of-chromosomes hinge domain-containing protein is required for RNA-directed DNA methylation. *Nature Genetics* **40**: 670–675.

Kloc A, Martienssen R. 2008. RNAi, heterochromatin and the cell cycle. *Trends in Genetics* **24**: 511–517.

Kloc A, Zaratiegui M, Nora E, Martienssen R. 2008. RNA interference guides histone modification during the S phase of chromosomal replication. *Current Biology* **18**: 490–495.

Kon T, Yoshikawa N. 2014. Induction and maintenance of DNA methylation in plant promoter sequences by apple latent spherical virus-induced transcriptional gene silencing. *Frontiers in Microbiology* **5**: 595.

Korner CJ, Pitzalis N, Pena EJ, Erhardt M, Vazquez F, Heinlein M. 2018. Crosstalk between PTGS and TGS pathways in natural antiviral immunity and disease recovery. *Nature Plants* **4**: 157–164.

Law JA, Jacobsen SE. 2010. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nature Reviews Genetics* **11**: 204–220.

Liu J, Feng L, Gu X, Deng X, Qiu Qi, Li Q, Zhang Y, Wang M, Deng Y, Wang E *et al.* 2019. An H3K27me3 demethylase-HSFA2 regulatory loop orchestrates transgenerational thermomemory in *Arabidopsis*. *Cell Research* **29**: 379–390.

Liu J, Zhang X, Yang Y, Hong N, Wang G, Wang A, Wang L. 2016. Characterization of virus-derived small interfering RNAs in *Apple stem grooving virus-infected* in vitro-cultured *Pyrus pyrifolia* shoot tips in response to high temperature treatment. *Virology Journal* **13**: 166.

Liu N, Xie K, Jia Q, Zhao J, Chen T, Li H, Wei X, Diao X, Hong Y, Liu Y. 2016. *Foxtail mosaic virus-induced* gene silencing in monocot plants. *Plant Physiology* **171**: 1801–1807.

Lloyd VK, Dyment D, Sinclair DA, Grigliatti TA. 2003. Different patterns of gene silencing in position-effect variegation. *Genome* **46**: 1104–1117.

Ma L, Huang X, Yu R, Jing XL, Xu J, Wu CA, Zhu CX, Liu HM. 2016. Elevated ambient temperature differentially affects virus resistance in two tobacco species. *Phytopathology* **106**: 94–100.

Matzke MA, Mosher RA. 2014. RNA-directed DNA methylation: an epigenetic pathway of increasing complexity. *Nature Reviews Genetics* **15**: 394–408.

Melnyk CW, Molnar A, Baulcombe DC. 2011. Intercellular and systemic movement of RNA silencing signals. *EMBO Journal* **30**: 3553–3563.

Molnar A, Csorba T, Lakatos L, Varallyay E, Lacomme C, Burgyan J. 2005. Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. *Journal of Virology* **79**: 7812–7818.

Otagaki S, Kawai M, Masuta C, Kanazawa A. 2011. Size and positional effects of promoter RNA segments on virus-induced RNA-directed DNA methylation and transcriptional gene silencing. *Epigenetics* **6**: 681–691.

Papikian A, Liu W, Gallego-Bartolome J, Jacobsen SE. 2019. Site-specific manipulation of *Arabidopsis* loci using CRISPR-Cas9 SunTag systems. *Nature Communications* **10**: 729.

Pooggin MM. 2013. How can plant DNA viruses evade siRNA-directed DNA methylation and silencing? *International Journal of Molecular Sciences* **14**: 15233–15259.

Pumplin N, Voinnet O. 2013. RNA silencing suppression by plant pathogens: defence, counter-defence and counter-counter-defence. *Nature Reviews Microbiology* **11**: 745–760.

Qu F, Ye X, Hou G, Sato S, Clemente TE, Morris TJ. 2005. RDR6 has a broad-spectrum but temperature-dependent antiviral defense role in *Nicotiana benthamiana*. *Journal of Virology* **79**: 15209–15217.

Ratcliff F, Martin-Hernandez AM, Baulcombe DC. 2001. Technical Advance: Tobacco rattle virus as a vector for analysis of gene function by silencing. *The Plant Journal* **25**: 237–245.

Ruiz MT, Voinnet O, Baulcombe DC. 1998. Initiation and maintenance of virus-induced gene silencing. *The Plant Cell* **10**: 937–946.

Schwach F, Vaistij FE, Jones L, Baulcombe DC. 2005. An RNA-dependent RNA polymerase prevents meristem invasion by potato virus X and is required for the activity but not the production of a systemic silencing signal. *Plant Physiology* **138**: 1842–1852.

Senthil-Kumar M, Mysore KS. 2011. New dimensions for VIGS in plant functional genomics. *Trends in Plant Science* **16**: 656–665.

Senthil-Kumar M, Mysore KS. 2011. Virus-induced gene silencing can persist for more than 2 years and also be transmitted to progeny seedlings in *Nicotiana benthamiana* and tomato. *Plant Biotechnology Journal* **9**: 797–806.

Senthil-Kumar M, Mysore KS. 2014. Tobacco rattle virus-based virus-induced gene silencing in *Nicotiana benthamiana*. *Nature Protocols* **9**: 1549–1562.

Szittya G, Silhavy D, Molnar A, Havelda Z, Lovas A, Lakatos L, Banfalvi Z, Burgyan J. 2003. Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. *EMBO Journal* **22**: 633–640.

Taochy C, Gursanscky NR, Cao J, Fletcher SJ, Dressel U, Mitter N, Tucker MR, Koltunow AMG, Bowman JL, Vaucheret H *et al.* 2017. A genetic screen for impaired systemic RNAi highlights the crucial role of DICER-LIKE 2. *Plant Physiology* **175**: 1424–1437.

Vaistij FE, Jones L, Baulcombe DC. 2002. Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. *The Plant Cell* **14**: 857–867.

Velázquez K, Renovell A, Comellas M, Serra P, García ML, Pina J, Navarro L, Moreno P, Guerri J. 2010. Effect of temperature on RNA silencing of a negative-stranded RNA plant virus: *Citrus psorosis virus*. *Plant Pathology* **59**: 982–990.

Voinnet O. 2008. Use, tolerance and avoidance of amplified RNA silencing by plants. *Trends in Plant Science* **13**: 317–328.

Wakasa Y, Kawakatsu T, Harada T, Takaiwa F. 2018. Transgene-independent heredity of RdDM-mediated transcriptional gene silencing of endogenous genes in rice. *Plant Biotechnology Journal* **16**: 2007–2015.

White JL, Kaper JM. 1989. A simple method for detection of viral satellite RNAs in small plant tissue samples. *Journal of Virological Methods* **23**: 83–93.

Yang J, Zhang T-Y, Liao Q-S, He L, Li J, Zhang H-M, Chen X, Li J, Yang J, Li J-B *et al.* 2018. *Chinese wheat mosaic virus-induced* gene silencing in monocots and dicots at low temperature. *Frontiers in Plant Science* **9**: 1627.

Yuan C, Li C, Yan L, Jackson AO, Liu Z, Han C, Yu J, Li D. 2011. A high throughput barley stripe mosaic virus vector for virus induced gene silencing in monocots and dicots. *PLoS ONE* **6**: e26468.

Zhang H, Lang Z, Zhu JK. 2018. Dynamics and function of DNA methylation in plants. *Nature Reviews Molecular Cell Biology* **19**: 489–506.

Zhang X, Zhang X, Singh J, Li D, Qu F. 2012. Temperature-dependent survival of *Turnip crinkle virus-infected* arabidopsis plants relies on an RNA silencing-based defense that requires DCL2, AGO2, and HEN1. *Journal of Virology* **86**: 6847–6854.

Zhong S-h, Liu J-z, Jin H, Lin L, Li Q, Chen Y, Yuan Y-x, Wang Z-y, Huang H, Qi Y-j *et al.* 2013. Warm temperatures induce transgenerational epigenetic release of RNA silencing by inhibiting siRNA biogenesis in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **110**: 9171–9176.

Zhong X, Du J, Hale CJ, Gallego-Bartolome J, Feng S, Vashisht AA, Chory J, Wohlschlegel JA, Patel DJ, Jacobsen SE. 2014. Molecular mechanism of action of plant DRM *de novo* DNA methyltransferases. *Cell* **157**: 1050–1060.

Zicola J, Liu L, Tanzler P, Turck F. 2019. Targeted DNA methylation represses two enhancers of FLOWERING LOCUS T in *Arabidopsis thaliana*. *Nature Plants* **5**: 300–307.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Progression of the *GFP* silencing in *N. benthamiana* 16c plants infected with TGS-inducing TRV-35S and PTGS-inducing TRV-GFP at 15/22/29°C.

Fig. S2 Raw bisulfite sequencing data of DNA methylation at *35S* promoter (from –208 to –89) in TRV-35S-infected *N. benthamiana* 16c plants and their progeny.

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Fig. S3 Examining the impact of temperature on the efficacy of T-DNA transfer and subsequent *GFP* expression in agroinfiltrated *N. benthamiana* leaves.

Fig. S4 Analysis of the effect of temperature on the efficiency of transgene-induced TGS and PTGS using agroinfiltration.

Fig. S5 Raw bisulfite sequencing data of DNA methylation at *35S* promoter (–208 to –89) in pDE-Ubi-35S-infiltrated *N. benthamiana* 16c leaves at 22 and 29°C.

Fig. S6 Time-course of TRV coat protein (TRV-CP) expression in inoculated and systemic leaves of TRV-infected *N. benthamiana* 16c plants at 29°C.

Fig. S7 Time-course of recombinant TRV-induced *GFP* silencing in *N. benthamiana* 16c plants at 22 and 29°C.

Fig. S8 Analysis of small RNAs in TRV-infected *N. benthamiana* 16c plants at 7 and 21 dpi.

Fig. S9 Size distribution of primary and secondary siRNAs in TRV-GFP-2M-infected 16c plants.

Fig. S10 The DNA methylation status of the target *GFP* coding region (+364 to +483) in the systemic leaves of TRV-GFP-infected *N. benthamiana* 16c plants at 22 and 29°C.

Methods S1 Construction of pDE vectors.

Methods S2 *Agrobacterium*-mediated transient assays in *N. benthamiana*.

Notes S1 Investigating the effect of temperature on transgene-induced PTGS and TGS using agroinfiltration.

Notes S2 Examining the effect of temperature on the initiation step of RdDM in virus-infected systemic tissues.

Table S1 Oligonucleotides used in this study.

Table S2 Summary of bisulfite sequencing data presented in Figs 2(d) and 3(c).

Table S3 Summary of bisulfite sequencing data presented in Fig. S4(d).

Table S4 Analysis of siRNA libraries.

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